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Substance P receptor blockade decreases stretch-induced lung cytokines and lung injury in rats

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Overdistension of lung tissue during mechanical ventilation causes cytokine release, which may be facilitated by the autonomic nervous system. We used mechanical ventilation to cause lung injury in rats, and studied how cervical section of the vagus nerve, or substance P (SP) antagonism, affected the injury. The effects of 40 or 25 cmH$_2$O high airway pressure injurious ventilation (HV$_{40}$ and HV$_{25}$) were studied and compared with low airway pressure ventilation (LV) and spontaneous breathing (controls). Lung mechanics, lung weight, gas exchange, lung myeloperoxidase activity, lung concentrations of interleukin (IL)-1β and IL-6, and amounts of lung SP were measured. Control rats were intact, others were bivagotomized, and in some animals we administered the neurokinin-1 (NK-1) receptor blocking agent SR140333. We first determined the durations of HV$_{40}$ and HV$_{25}$ that induced the same levels of lung injury and increased lung contents of IL-1β and IL-6. They were 90 min and 120 min, respectively. Both HV$_{40}$ and HV$_{25}$ increased lung SP, IL-1β and IL-6 levels, these effects being markedly reduced by NK-1 receptor blockade. Bivagotomy reduced to a lesser extent the HV$_{40}$- and HV$_{25}$-induced increases in SP but significantly reduced cytokine production. Neither vagotomy nor NK-1 receptor blockade prevented HV$_{40}$-induced lung injury but, in the HV$_{25}$ group, they made it possible to maintain lung injury indices close to those measured in the LV group. This study suggests that both neuronal and extra-neuronal SP might be involved in ventilator-induced lung inflammation and injury. NK-1 receptor blockade could be a pharmacological tool to minimize some adverse effects of mechanical ventilation.

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Abbreviations ABP, arterial blood pressure; ARDS, acute respiratory distress syndrome; BW, body weight; C_{rsi}, inspiratory compliance of the respiratory system; HV, high-pressure ventilation; IL, interleukin; LIP, lower inflection point; LV, low-pressure ventilation; MPO, myeloperoxidase; NK-1, neurokinin-1; P_{aw}, airway pressure; SP, substance P; SPB, substance P blockade; TFA, trifluoroacetic acid; VALI, ventilator-associated lung injury; VILI, ventilator-induced lung injury.

Introduction

Strategies of mechanical ventilation with small tidal volume and limited airway pressures are recommended by experts' consensus to protect patients with acute respiratory distress syndrome (ARDS) (International consensus conferences in intensive care medicine, 1999) against ventilator-induced or -associated lung injury (VILI/VALI), because they are supposed to minimize lung stretch. However, despite the use of reduced-volume mechanical ventilation, the risk of VALI persists in ARDS patient who have heterogeneous lungs, regionally exposed to cyclic alveolar overdistension, even at the recommended plateau pressure lower than 30 cmH$_2$O (Gattinoni *et al.* 2001). In alveoli submitted to overdistension, the mechanical cell stress can lead to the subsequent activation of the inflammatory innate immune system with cytokine release known as biotrauma (Dos

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Santos & Slutsky, 2000). To assess the pathophysiology of VILI, animal models of lung injury induced by high-pressure/high-volume mechanical ventilation in previously intact lungs have been extensively used by several authors expert in this field (Webb & Tierney, 1974; Dreyfuss *et al.* 1985; Wilson *et al.* 2003; Sinclair *et al.* 2004; Frank *et al.* 2006). Both experimental and clinical studies agree that cytokines play a crucial role in VILI (Narimanbekov & Rozycki, 1995; Tremblay *et al.* 1997; Imai *et al.* 1999; Ranieri *et al.* 1999; Wilson *et al.* 2003). The release of cytokines recruits and activates leukocytes in the lungs, representing the hallmarks of lung injury. The magnitude of cytokine release correlates with that of the lung stretch due to the ventilator (Tremblay *et al.* 1997; Ranieri *et al.* 1999; Wilson *et al.* 2003). It was shown that cytokine receptor blockade or cytokine gene deficiency afforded protection against acute lung injury (Narimanbekov & Rozycki, 1995; Imai *et al.* 1999; Frank *et al.* 2008; Klein *et al.* 2008).

Numerous biological and cellular events reported in VILI have been highlighted but data on their modulation by the neuro-immune system are very scarce. We only found one study in mice showing that selective sensory C fibre denervation with capsaicin or targeted deletion of the preprotachykinin A gene decreased substance P (SP) immunoreactivity in alveolar macrophages. It also reduced the lung cytokine response to injurious mechanical ventilation (Chavolla-Calderon *et al.* 2003). These data suggest that SP and lung-borne cytokines may interact in the pathophysiology of VILI. SP is a major tachykinin of the non-adrenergic non-cholinergic system in the afferent vagal C fibres innervating the lungs, involved in bronchial and microvascular tone (Barnes, 1986). SP also exerts inflammatory actions via its promoting effect on cytokine release (Maggi, 1997). Increased lung SP content has been reported in several animal models of acute lung injury (Bhatia *et al.* 1998; Lau & Bhatia, 2006; Puneet *et al.* 2006; Sio *et al.* 2008) and also in ARDS patients (Espiritu *et al.* 1992; Bhatia & Moochhala, 2004). We have already shown that the SP concentration increased in the cervical vagus nerve during moderate-volume mechanical ventilation of rabbits with previously intact lungs (Balzamo *et al.* 1996). It is thus possible to imagine that SP is released in the lung by vagal afferents and/or macrophages and participates in the mechanism of VILI.

The aim of the present work was to test the role of SP, through the activation of its neurokinin-1 (NK-1) receptor, in ventilator-induced lung cytokine release and lung injury in rats with previously intact lungs. We chose to ventilate the rats with high airway pressure to elicit the largest amount of cytokines (Tremblay *et al.* 1997), while a low-pressure (6 cmH_2O P_{aw}) does not (Chavolla-Calderon *et al.* 2003). Thus, we hypothesised that high-pressure ventilation should highlight the neurokinin control of lung cytokine release.

Methods

Animal care and general preparation

The article by G. B. Drummond (Drummond, 2009) was read carefully to ensure that our experiments complied with the policies and regulations it describes. The protocol also conformed to the guidelines laid out in the *Guide for the Care and Use of Laboratory Animals* and the experiments were performed within the requirements of the ethics committee of the Jean Roche Institute.

Eighty-five adult Sprague–Dawley rats were studied (mean body weight (BW) 355 ± 6 g). Animals were anaesthetized with an intraperitoneal mixture of sodium pentobarbitone (20 mg kg^{-1}) and ethyl carbamate (0.5 g kg^{-1}). Before skin incision in spontaneously breathing animals, additional doses of anaesthetic agents were given when necessary on the basis of the response to tail-pinch. The left carotid artery was catheterized for arterial blood pressure (ABP) and heart rate measurements (electromanometer; Statham P23 Db, Puerto Rico, USA). An external jugular vein was cannulated and a vascular volume expansion with 1.5 ml of saline was performed to ensure an initial ABP above 140 mmHg. A heating pad enabled the rectal temperature to be maintained in the range 37–38°C. A tracheotomy was performed and a side port of the tracheal cannula was connected to a differential electromanometer to measure the airway pressure (P_{aw}). Throughout and after the operative procedure, the adequacy of the level of anaesthesia was judged from the changes in blood pressure and heart rate, the changes in these variables governing the injection of supplementary doses of anaesthetics. In all animals, both cervical vagus nerves were dissected and exposed for further surgical section or as a sham comparison.

In a control group, the rats were tracheotomized (controls, $n = 4$) and remained under general anaesthesia while they breathed spontaneously for a 120 min period. Their vagus nerves were left intact. All other rats were mechanically ventilated. At the end of the experiments, animals were killed by an intravenous overdose of 5% sodium pentobarbitone.

Mechanical ventilation

Mechanical ventilation was delivered via a Harvard Rodent Ventilator Model 683 volumetric pump delivering room air at a rate of 70 breaths min^{-1} and we added a 1 cmH_2O end-expiratory pressure. Neuromuscular blocking agent (cisatracurium besilate 0.2 mg kg^{-1}) was injected to avoid increased P_{aw} due to superimposed spontaneous breathing.

The pump volume was randomly set to ensure either low-pressure ventilation (8 cmH_2O P_{aw}; 7–9 ml kg^{-1}

tidal volume) (LV group) or two different levels of high-pressure ventilation, i.e. a 25 cmH_2O P_{aw} (20–25 ml kg^{-1} tidal volume, HV_{25} groups) or a 40 cmH_2O P_{aw} (30–40 ml kg^{-1} tidal volume, HV_{40} groups). In the HV_{25} and HV_{40} groups, CO_2 was added to ambient air to maintain normocapnia despite hyperventilation. Arterial blood gas analyses were repeatedly performed throughout the experiments (Radiometer ABL 330, Copenhagen), and P_{aw} and ABP were continuously recorded (Gould TA 4000, Ballinviliers, France).

A preliminary study was performed in 24 animals ventilated with HV_{40}, and 22 other animals with HV_{25}, for 30, 60, 90 or 120 min (the number of animals at each epoch is indicated in Fig. 1 legend). This allowed us to determine the duration of exposure to mechanical ventilation giving the highest cytokine levels combined with more than 50% survival rate and an associated lung injury in 100% of cases. It showed that at 90 min, the exposure to HV_{40} ventilation induced a 10-fold increase in IL-1β, a 30-fold increase in IL-6, and a 60% survival rate. Acute lung injury was present in all cases. For the same epoch, the exposure to HV_{25}, despite a high survival rate, induced lung injury in only 50% of the animals. At 120 min of HV_{25}, although the survival rate was still high (90%), all rat lungs were injured, and IL-1β and IL-6 levels were not far from those measured at 90 min in HV_{40} (Fig. 1). Based on these data, we decided to explore the consequences of NK-1 receptor blockade on the lung production of SP and cytokines at 90 min during HV_{40}, and at 120 min during HV_{25}.

Animal subgroups in the main study

Only animals that completed the protocol were analysed in the main study consisting of the control group of intact rats breathing spontaneously (controls, $n = 4$) and seven groups of mechanically ventilated rats:

LV group for 120 min. $n = 7$

HV_{40} for 90 min. One group received only isotonic saline (HV_{40}-sham group, $n = 10$), a second group was bivagotomized (HV_{40}-vagotomy, $n = 7$), and a third group was pretreated with NK-1 receptor blockade (HV_{40}-SPB group, $n = 7$).

HV_{25} for 120 min. One group received only isotonic saline (HV_{25}-sham group, $n = 10$), a second group was bivagotomized (HV_{25}-vagotomy, $n = 7$) and a third group was pretreated with NK-1 receptor blockade (HV_{25}-SPB group, $n = 7$).

Chemicals

Isotonic saline solution was NaCl 0.9%. SR140333, a very potent and selective NK-1 receptor antagonist (Emonds-Alt *et al.* 1993), was generously provided by Dr Xavier Emonds-Alt (Sanofi Aventis Recherche et Développement, Montpellier, France). It was dissolved in dimethyl sulfoxide and aliquots stored at −20°C. At the time of the experiment, 1 mg kg^{-1} was injected intraperitoneally 30 min before HV, this dosage being sufficient to reduce acute lung injury-like effects in rats (Wong *et al.* 2004).

Measurements of pulmonary mechanics

After the animals were killed, the lungs were ventilated with 8 ml kg^{-1} tidal volume for 10 cycles to standardize the lung volume history, after which the volume–pressure relationship was determined. In oedematous lungs, the volume–pressure relationship characteristics were determined according to a method fitted to mechanically ventilated oedematous lungs (Martin-Lefèvre *et al.* 2001): inflated volumes ranged from 0 to 10 ml, the

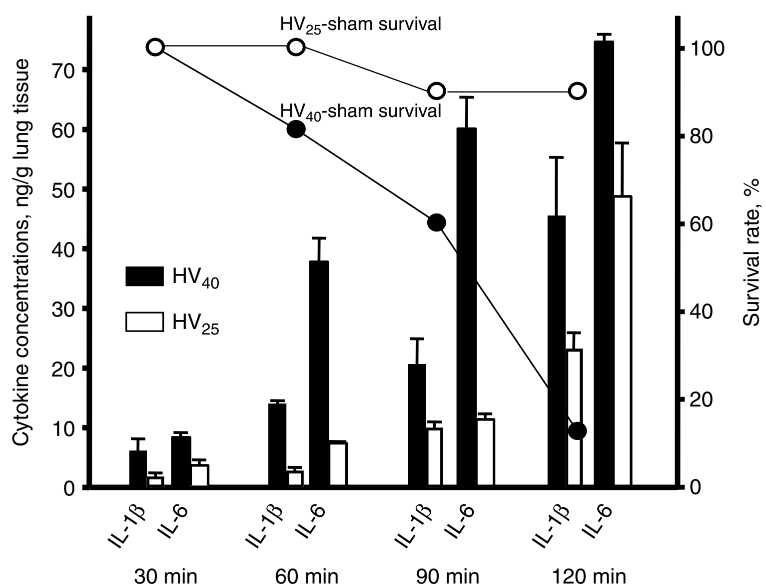

Figure 1. The preliminary study
IL-1β and IL-6 concentrations in lung tissue from 8 series of rats ventilated with airway pressures of 40 (HV_{40}) or 25 (HV_{25}) cmH_2O for 30, 60, 90 or 120 min. $n = 3$–7 observations per series for 30 and 60 min epochs and 10 observations per series for 90 and 120 min epochs. The corresponding survival rate, at each period, was calculated as the ratio of rats alive to the total number of rats. Bars represent mean ± S.E.M. cytokine concentrations (left axis); lines represent the survival rates (right axis), filled circles/bars correspond to HV_{40} ventilation, open circles /bars represent HV_{25} ventilation.

inspiratory compliance of the respiratory system (C_{rsi}) was calculated as the slope of the least-squares regression of the volume–pressure relationship, situated between the bottom of the curve and the lower inflection point (LIP) and was indexed to the body weight (C_{rsi}/BW). In animals having normal lung compliance, no LIP could be determined and the slope of the least-squares regression of the volume–pressure relationship was determined from the initial linear segment of the curve. Then the trachea was clamped at end-inspiration (inflated at 8 ml kg^{-1}) before sternotomy and the lungs were removed *en bloc* with the heart, separated from other thoracic organs and weighed. They were divided into three fragments and frozen to −80°C for further biochemical assays.

Lung cytokine assays

A lung sample from each animal was homogenized in 0.01 M phosphate buffer saline, pH 7.4, according to a weight/volume ratio of 1/4 with an Ultra-Turrax T25 basic disperser (Ika-Werke, Staufen, Germany) at 24,000 rotations per minute. The resultant mixtures were centrifuged (10,000 *g* at 4°C for 15 min), and the cytokine levels were measured in the supernatant with sensitive Enzyme-Linked ImmunoSorbent Assay (ELISA) kits (Pierce Endogen, ER2IL1B for IL-1β, ER2IL6 for IL-6, supplied by Thermo Fisher Scientific, Perbio Science France SAS, Brebières, France). The limits of detection of IL-1β and IL-6 assays were 12 and 16 pg ml^{-1}, respectively, on the standard curve. All measurements were made in duplicate by spectrophotometry on a StatFax 3200 microplate reader (Awareness Technology Inc., Palm City, FL, USA).

SP extraction and detection

A further series of lung samples was homogenized in a solution containing 4 ml of 0.1 N acetic acid, 200 μl of 5% (w/v) ethylenediamine tetra-acetic acid, and 20 μl of 1 mM aprotinin with an Ultra-Turrax TP 18/10 disperser. After centrifugation (10,000 *g* at 4°C for 15 min), the supernatants were stored at −80°C. At the time of analysis, SP extraction was performed on a 6 ml C18 solid phase extraction cartridge (Cayman Chemical Company, Ann Arbor, MI, USA). The extracts were then lyophilized and quantified. The scattering of data measured by S.D. coefficient corresponds to that of repeated measurements in the same aliquot constituted by 1 mg of dry lung extract collected from all individuals in each group. Aliquots of extracts (1 mg per run) were analysed by analytical C18 reversed-phase high-performance liquid chromatography (HPLC), (C18 Monolithic 2 μm, 100 mm × 4.6 mm; Onyx) by means of a 60 min linear gradient of 0.08% (v/v) trifluoroacetic acid (TFA) 0% to 40% acetonitrile in 0.1% (v/v) TFA/H_2O at a flow rate of 1 ml min^{-1} ($\lambda = 230$ nm). The presence of substance P was checked by molecular mass analysis using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. One milligram of SP acetate salt hydrate (Sigma-Aldrich, France) was used as control. The area under the SP peak chromatogram was expressed in arbitrary units and used for assessment of SP amounts.

Lung myeloperoxidase (MPO)

Other lung samples were homogenized in 0.5% hexadecyltrimethylammonium bromide in 10 mM 3-(*N*-morpholino) propanesulfonic acid, according to a weight/volume ratio of 1/4 with an Ultra-Turrax T25 basic disperser. The resultant mixtures were then centrifuged (15,000 *g* at 4°C for 40 min) and 100 μl of supernatant were mixed with 2.9 ml of a solution containing 1% (w/v) dimethoxybenzidine and 1 mM hydrogen peroxide. After a 30 min incubation period, the reaction was stopped by 200 μl of 3 M HCl and the resulting MPO activity was measured with a spectrophotometer (Spectronic Genesys 2, Milton Roy Company, Rochester, NY, USA). One unit of MPO activity was arbitrarily defined as the amount of enzyme necessary to catalyse an increase in absorbance of 1.0 at 410 nm per minute at 37°C.

Statistics

The SigmaStat 3.0 program (Sigma Company, Erkrath, Germany) was used. Data were tested for normal distribution with the Kolmogorov–Smirnov test. When the data distribution of continuous variables was not normal, median and quartile values are given in the text and individual data or box plots (median and quartile values) are presented in the figures. When data were normally distributed, we use mean ± S.E.M. in the text and figures.

For most of the temporally repeated data, changes over time were assessed in each group using a repeated measures ANOVA followed by a *post hoc* Holm–Sidak comparison *versus* baseline. Comparisons were made between HV_{40}-SPB or HV_{40}-vagotomy group *versus* the HV_{40}-sham or between HV_{25}-SPB or HV_{25}-vagotomy group *versus* the HV_{25}-sham group using Student's *t* test or the non-parametric Mann–Whitney rank sum test. A *P* value ≤ 0.05 was used to determine statistical significance.

Results

Effects of HV_{25} and HV_{40} on lung function in rats

No significant changes in respiratory (C_{rsi}/BW, P_{aO_2}, P_{aCO_2}) and circulatory (ABP) variables were measured in controls, i.e. spontaneously breathing rats, or in the LV group. In addition, both levels of high-pressure ventilation (HV_{40} and HV_{25}) induced a lung injury attested by the occurrence of hypoxaemia (Fig. 2*A*). Compared to the HV_{40}-sham group, the HV_{25}-sham group had a similar but delayed hypoxaemia (HV_{25}-sham at 120 min *versus* HV_{40}-sham at 90 min: $P_{aO_2} = 63 \pm 8$ *versus* 59 ± 5 mmHg). Figure 2*B* shows the increases in P_{aw} in the HV_{40}-sham and HV_{25}-sham groups. Although the mean amount of total fluid administration did not differ between groups, the ABP decrease was significantly accentuated in the HV_{40}-sham animals compared to the HV_{25}-sham animals from the 45th minute of exposure to HV ($P < 0.01$) (Fig. 2*C*). The mean P_{aCO_2} did not significantly differ between the LV, HV_{40}-sham and HV_{25}-sham groups (40 ± 2, 42 ± 1 and 47 ± 3 mmHg, respectively). The mean C_{rsi}/BW value measured after 90 min of exposure to HV_{40} was as low as that measured after 120 min of exposure to HV_{25} (median (25–75 percentiles): 0.20 (0.18–0.29) and 0.21 (0.19–0.25) ml cmH_2O^{-1} kg^{-1}, respectively). The lung weight indexed to body weight did not differ significantly (mean ± S.E.M.: 5.00 ± 0.20 *versus* 6.08 ± 0.49 g (kg BW)$^{-1}$ for HV_{40}-sham *vs.* HV_{25}-sham groups).

Figure 3 shows the absorbance intensity peaks in representative extracts of the different groups: SP was nearly undetectable by HPLC in lyophilized lung extracts in controls and LV animals, whereas it was much increased in rats exposed to high-pressure ventilation. In Fig. 4, SP amounts are expressed as the mean (± S.E.M.) areas under the SP peak chromatograms and compared between groups. The highest SP amounts were detected in the HV_{40}-sham group. Bivagotomy moderately decreased the SP release in the HV_{40} and HV_{25} groups, whereas NK-1 receptor blockade was much more effective. As shown in Figs 3 and 4, NK-1 receptor blockade resulted in undetectable amounts of SP in the HV_{25}-SPB lungs.

With respect to cytokines, Fig. 5 shows that HV_{40} ventilation for a 90 min period induced a significant increase in lung IL-1β and IL-6 concentrations compared to control and LV groups. In both high-ventilation groups, bivagotomy as well as the NK-1 receptor blockade reduced the lung concentrations of IL-1β and more importantly of IL-6.

Effects of vagotomy or NK-1 receptor blockade on ventilator-induced lung injury

The NK-1 receptor blockade resulted in a better survival rate at the 90th minute of exposure to HV_{40} (1 of 7 rats died in the HV_{40}-SPB group *versus* 4 of 10 in the HV_{40}-sham group). Similarly, 1 of 10 rats died spontaneously in the HV_{25}-sham group whereas none of 7 rats died in the HV_{25}-SPB group. By contrast, vagotomy did not protect the animals from premature death during HV_{40} ventilation (7/10 animals). One of seven rats died prematurely (90th minute) in the HV_{25}-vagotomy group.

With regard to the lung injury indices, vagotomy or pretreatment with the NK-1 receptor blocking agent was inefficient during HV_{40} ventilation (C_{rsi}/BW medians were: 0.21 (0.18–0.25) and 0.33 (0.26–0.35) ml cmH_2O^{-1} kg^{-1}, respectively; mean lung weight indexed to body weight was 6.77 ± 0.27 and 6.86 ± 0.59 g kg^{-1}, respectively). During HV_{25}

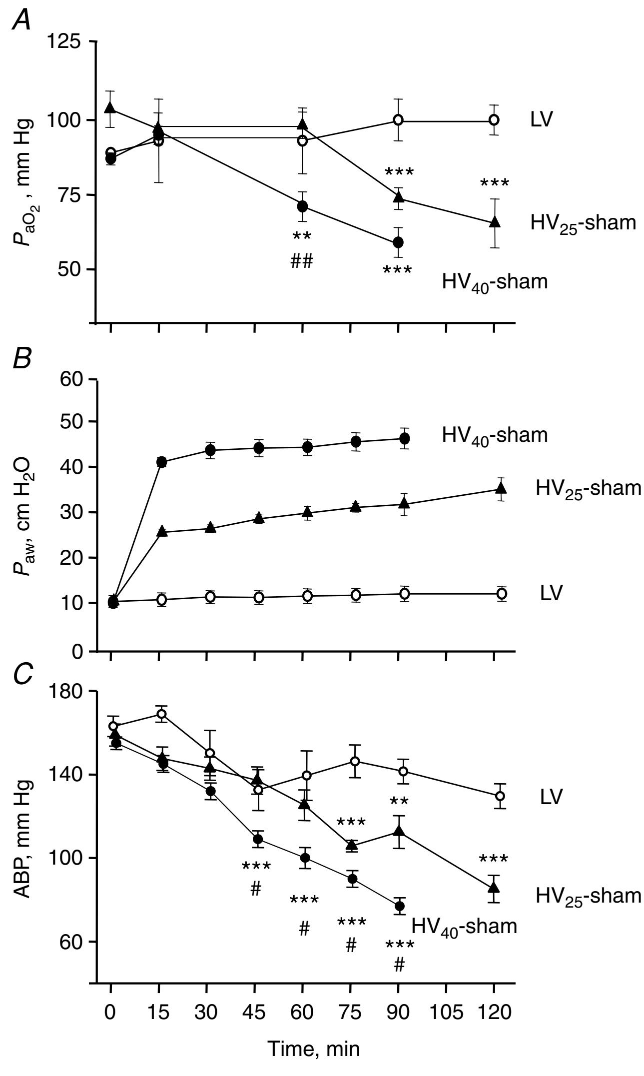

Figure 2
Time course of P_{aO_2} (*A*), airway pressure (P_{aw}) (*B*), and arterial blood pressure (ABP) (*C*), in animals receiving low-pressure ventilation (LV), high-pressure ventilation at 40 cmH_2O airway pressure (HV_{40}-sham) and high-pressure ventilation at 25 cmH_2O airway pressure (HV_{25}-sham). Asterisks indicate significant differences *versus* baseline (**$P < 0.01$, ***$P < 0.001$). # indicates significant differences between HV_{40}-sham and HV_{25}-sham groups (#$P < 0.05$, ##$P < 0.01$).

ventilation, however, vagotomy or NK-1 receptor blockade dramatically improved all the measured indices of lung injury (Fig. 6). Indeed, the volume–pressure curves clearly show that the shift to the right and the LIP observed in the HV_{25}-sham group were not present (HV_{25}-SPB group) or minimized (HV_{25}-vagotomy group). Also, the C_{rsi}/BW medians were markedly higher in the HV_{25}-vagotomy and HV_{25}-SPB groups than in the

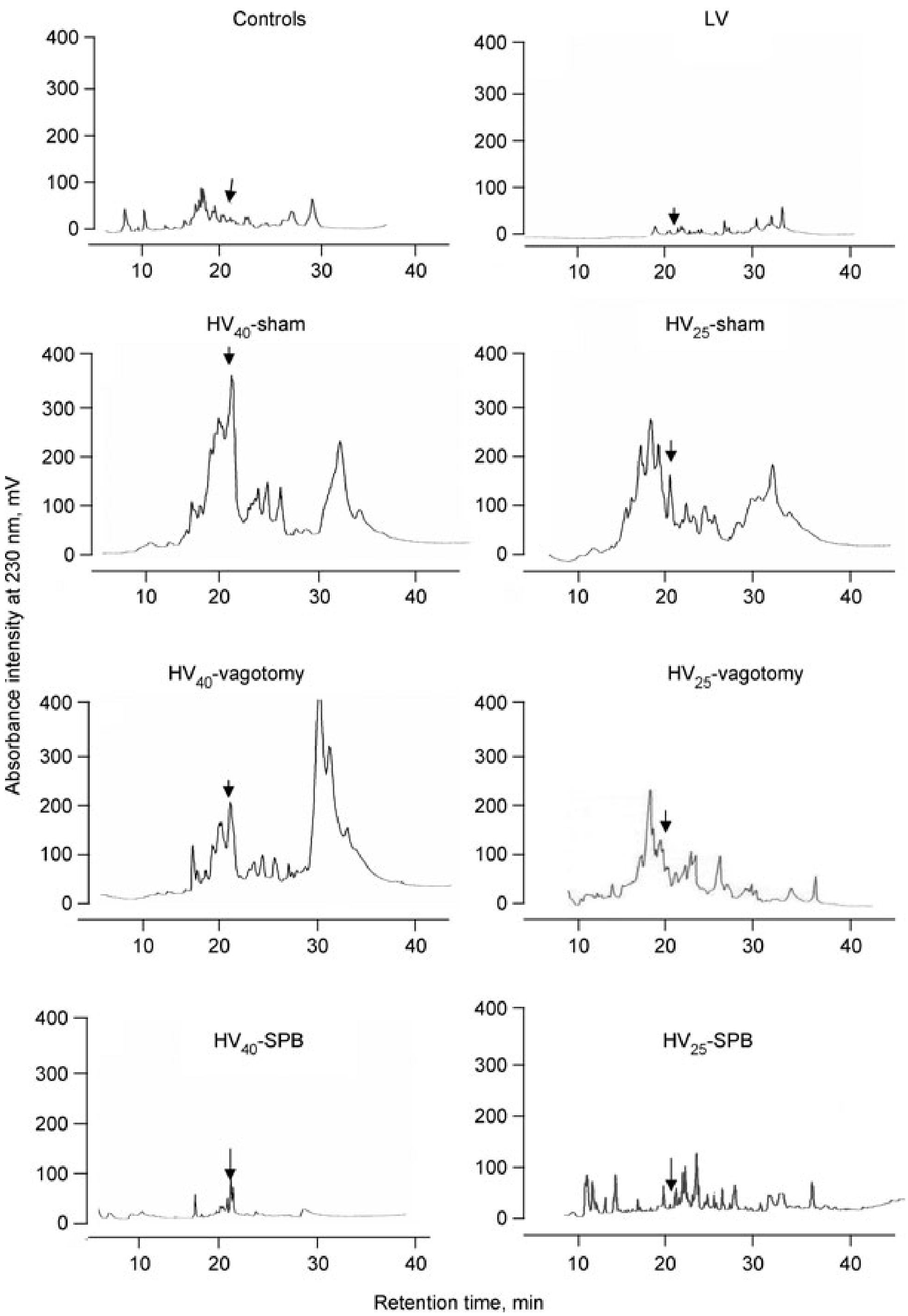

Figure 3
Examples of HPLC profiles expressed as absorbance intensity at 230 nm (mV) *versus* retention time (minutes) chromatograms in each rat group: spontaneously breathing rats (Controls), low-pressure ventilation (LV), rats ventilated with 25 cmH_2O (HV_{25}-sham) or 40 cmH_2O airway pressure (HV_{40}-sham), bivagotomized rats (HV_{40}-vagotomy and HV_{25}-vagotomy) and rats pretreated with NK-1 receptor blockade (HV_{40}-SPB, HV_{25}-SPB). The arrows indicate the peaks corresponding to substance P.

HV_{25}-sham group (1.68 (1.44–1.79) and 1.66 (1.60–1.68) *versus* 0.20 (0.18–0.29) ml cmH_2O^{-1} kg^{-1}, respectively, $P < 0.001$).

HV_{25}-vagotomy and HV_{25}-SPB animals did not develop hypoxaemia (at the end of the experiment, $P_{aO_2} = 93 \pm 5$ and 107 ± 6 mmHg in the HV_{25}-vagotomy and HV_{25}-SPB groups *versus* 64 ± 8 mmHg in the HV_{25}-sham group, $P < 0.001$).

The lung weight indexed to body weight and the MPO values measured at the end of the experiments, which were high in the HV_{25}-sham group, were low in the HV_{25}-SPB groups, close to their respective values measured in the LV group. Only lung weight was significantly decreased in the HV_{25}-vagotomy group (Fig. 6).

Discussion

The main findings of the present study are that SP and the pro-inflammatory cytokines IL-1β and IL-6 are released in the lung parenchyma during the ventilator-induced lung stretch, and that both vagotomy and NK-1 receptor blockade minimized the stretch-induced lung cytokine production. This highlights the role of SP in lung cytokine upregulation induced by high-pressure ventilation. In addition, although ineffective during HV_{40}, vagotomy or NK-1 receptor blockade prevented the lung injury induced by HV_{25}, nearly suppressing the variations in lung mechanics, lung weight and MPO present in the HV_{25}-sham group.

The mechanisms of HV-induced cytokine release are only suspected. The lung stretch induces a mechanical disruption of the alveolar–capillary barrier, causing an intense pulmonary oedema (Dreyfuss *et al.* 1985). The direct contact between the basement membrane and the circulating immunocompetent cells might be responsible for their activation (Dreyfuss & Saumon, 1998). This may have contributed to increasing the lung cytokine production and MPO activity in our HV_{40} and HV_{25} ventilation models. We limited our study to the measurements of IL-1β and IL-6 lung cytokines because both cytokines have been shown to respond strongly to the ventilation of previously healthy lungs (Tremblay *et al.* 1997, 2002; Brégeon *et al.* 2002, 2004; Chu *et al.* 2004; Frank *et al.* 2008). Moreover, these cytokines are released within the first 60–120 min of HV (Stuber *et al.* 2002; Rich *et al.* 2003), a delay that corresponds to the duration of our experiments. Also, some reports have suggested that both IL-1β and IL-6 might be involved in vagally mediated pro-inflammatory neuro-immune interactions (Lotz *et al.* 1988; Linard *et al.* 2005; Yu *et al.* 2007).

Vagally mediated neuro-immune interactions result from complex mechanisms since pro- as well as anti-inflammatory pathways coexist in the same nerve, both being possibly affected by vagotomy. Among mediators contained in the vagus nerve, neurokinins (afferent fibres) are known to be potent inducers of inflammation (Barnes, 1986) but acetylcholine can also worsen lung inflammation (Lutz & Sulkowski, 2004; McQueen *et al.* 2007). The involvement of multiple vagal pro-inflammatory mediators may explain why vagotomy was more effective on lung cytokines than on SP reduction in our study.

SP is considered an important inflammatory substance in pulmonary diseases combining inflammation and lung distension, such as asthma (Barnes, 1986; Nieber *et al.* 1992; Chu *et al.* 2000). When activated by prolonged inflammatory stimuli, the bronchopulmonary vagal C fibres, which represent the major source of pulmonary SP, can retrogradely release SP into the innervated tissues (Barnes, 1986; Lundberg & Saria, 1987). Once released, SP can stimulate the immunocompetent cells to produce inflammatory cytokines, including IL-1β and IL-6 (Lotz *et al.* 1988). It is therefore likely that the stretch-induced inflammation might upregulate the lung SP release, for example through vagus nerve terminals (Yu *et al.* 2007), and that, in its turn, SP might promote the lung cytokine response, constituting a vicious cycle. On the other hand, SP release can also be triggered by C terminal fibre depolarization, which depends on the amplitude of lung inflation (Paintal, 1969; Delpierre *et al.* 1981). Thus, lung SP may increase during lung stretch

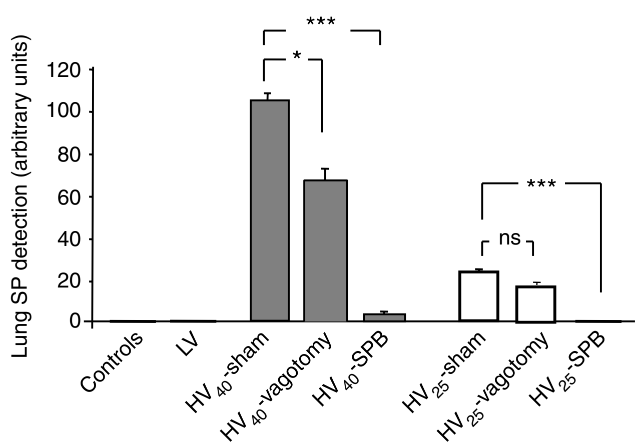

Figure 4. Quantification of SP detection by measurement of the area under the SP peaks in lung extracts
Controls: rats breathing spontaneously; LV: rats ventilated with low-pressure ventilation; HV_{40}: rats with high-stretch ventilation using 40 cmH_2O P_{aw}, injected with saline (HV_{40}-sham) or bivagotomized (HV_{40}-vagotomy), or after pretreatment with NK-1 receptor blockade (HV_{40}-SPB); HV_{25}: rats with high-stretch ventilation using 25 cmH_2O P_{aw}, injected with saline (HV_{25}-sham) or bivagotomized (HV_{25}-vagotomy), or after pretreatment with NK-1 receptor blockade (HV_{25}-SPB). Data were normally distributed. Bars represent mean $\pm$ S.E.M. Asterisks indicate significant intergroup differences (*$P < 0.05$; ***$P < 0.001$).

before cytokines. Since the SP half-life is less than 5 min (Blumberg & Teichberg, 1979) whereas cytokines persist for hours, it is likely that IL-6 and IL-1β accumulated over time in the HV_{25}-sham group whereas SP amounts were moderate, leading, at the end, to levels of cytokines as high as those of the HV_{40}-sham group. In addition, it was demonstrated that stretching lung parenchyma directly upregulates the cytokine production by the macrophages, endothelial cells and pneumocytes (Pugin *et al.* 1998; Vlahakis & Hubmayr, 2000; Tremblay *et al.* 2002), here adding probably to SP-related cytokine production and injury. Higher SP amounts in the HV_{40}-sham group may have participated in the observed haemodynamic failure in the HV_{40}-sham group. Also, high SP amounts may have provoked a bronchospasm, but if present, this was probably a minor effect in our model since the very high airway pressure imposed by the mechanical ventilation was prevalent.

In a previous mouse study, Chavolla-Calderon and coworkers (Chavolla-Calderon *et al.* 2003) showed that the absence of vagal C fibres, induced by a neonatal injection of capsaicin or a congenital deficiency in the preprotachykinin A gene, was associated with a decreased SP immunoreactivity in alveolar macrophages and a reduced lung cytokine response to injurious mechanical ventilation (16–17 cmH_2O P_{aw}). Interestingly, in our study the reduction of the HV-induced SP response by pharmacological blockade was significantly more effective than by vagotomy. This could be the consequence of extraneuronal sources of SP, able to interact synergistically with their neuronal sources (Chavolla-Calderon *et al.* 2003). Indeed, extra-neuronal SP was found in lung-resident macrophages and the circulating leukocytes which both express NK-1 receptors, suggesting the possibility of an autocrine control (Pascual & Bost, 1990; Killingsworth *et al.* 1997). It must be

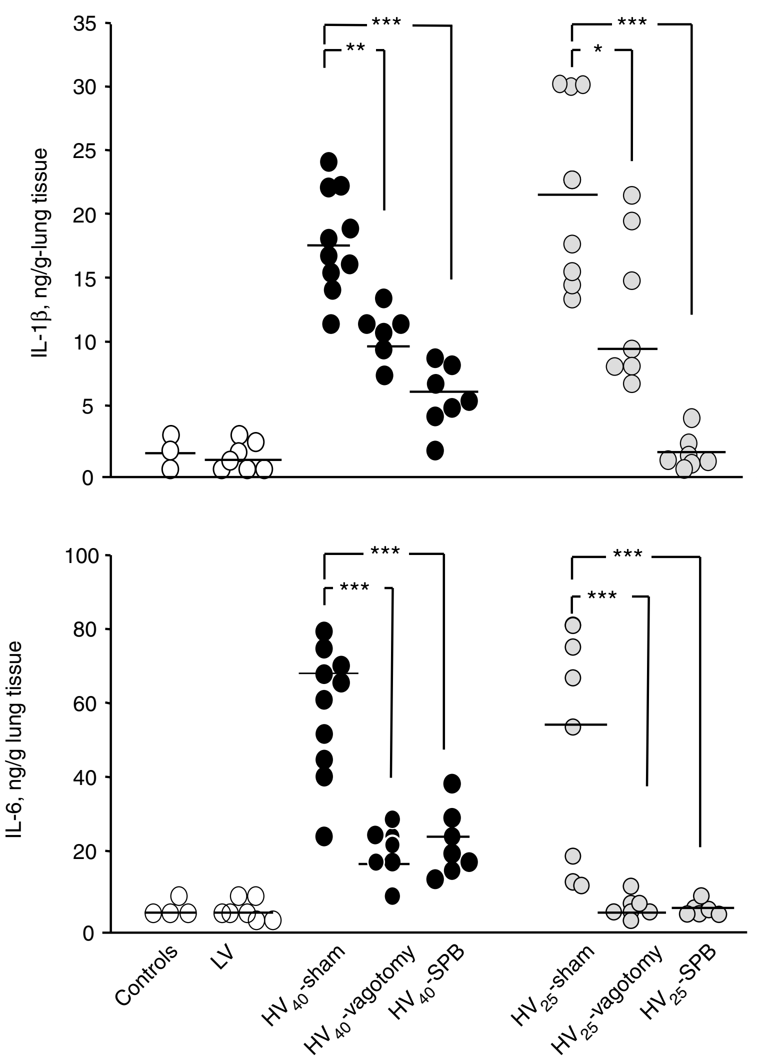

Figure 5. Individual (circles) and median (horizontal bars) lung concentrations of IL-1β and IL-6 measured by ELISA
Controls: rats breathing spontaneously; LV: rats ventilated with low-pressure ventilation; HV_{40}: rats with high-stretch ventilation using 40 cmH_2O P_{aw}, injected with saline (HV_{40}-sham) or bivagotomized (HV_{40}-vagotomy), or after pretreatment with NK-1 receptor blockade (HV_{40}-SPB); HV_{25}: rats with high-stretch ventilation using 25 cmH_2O P_{aw}, injected with saline (HV_{25}-sham) or bivagotomized (HV_{25}-vagotomy), or after pretreatment with NK-1 receptor blockade (HV_{25}-SPB). Asterisks indicate significant intergroup differences using the non-parametric test ($^*P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$).

underlined that the lung cytokine contents were reduced but not abolished by vagotomy or pharmacological blockade, especially during HV_{40} ventilation. This may be explained by the above-cited multifactorial sources of cytokine release. During the most important lung stretch (HV_{40} ventilation), intense cardiopulmonary interactions (and possibly high SP levels) led to haemodynamic failure with possible cytokine production by hypoperfused organs. Some circulating cytokines may have contributed to persistent high levels of pulmonary cytokines during HV_{40}-vagotomy and HV_{40}-SPB. Also, as previously documented with electronic microscopy, HV_{40} ventilation causes such distension that epithelial and endothelial breaks occur (Dreyfuss & Saumon, 1998): these lesions are probably out of reach of any treatment and result in the persistence of lung injury despite reduction in lung cytokines in our HV_{40} vagotomy and HV_{40}-SPB groups.

The present animal observations have some clinical interest because we showed that NK-1 receptor blockade prevented lung injury in the HV_{25}-SPB group. We speculate that this protective effect may act in part via the reduction in cytokine levels. Moreover, the beneficial effect of NK-1 receptor blockade in this group may result partly from its anti-oedematous action, SP being known to cause neurogenic lung oedema (Wong *et al.* 2004). In summary, the present rat study demonstrates the roles of SP, and of the activation of its NK-1 receptor, in the mechanism of stretch-induced lung inflammation and injury. The efficacy of NK-1 receptor blockade to afford protection against VILI would suggest the use of a new pharmacological tool in ARDS patients in whom

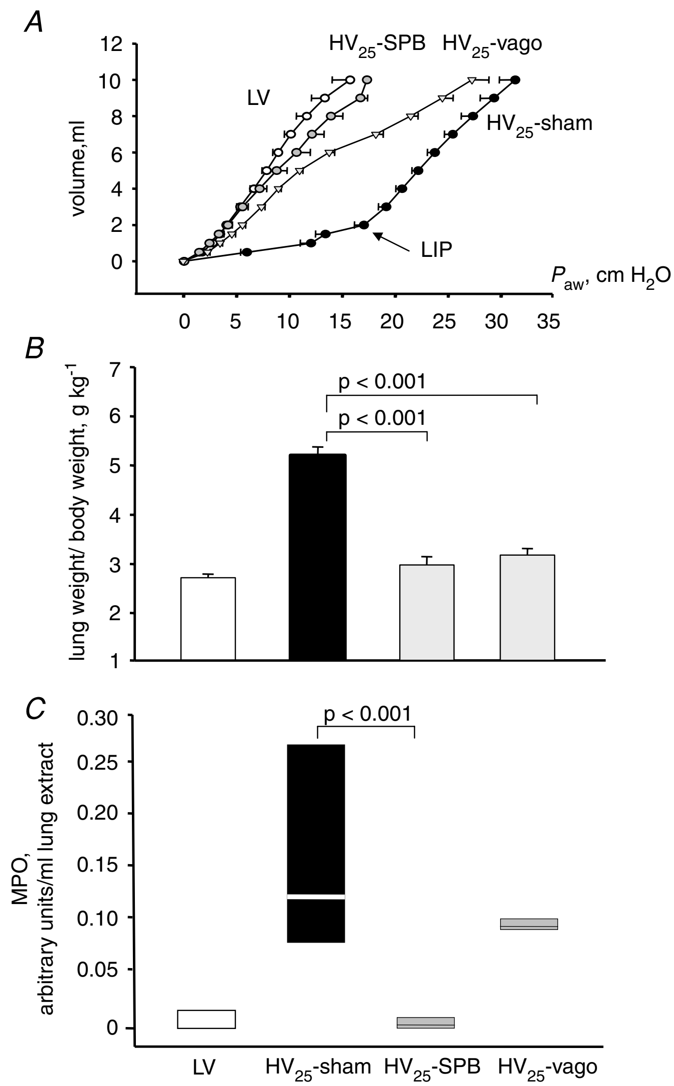

Figure 6

A, comparison of respiratory system pressure–volume curves during inflation, showing the absence (NK-1 receptor blockade), or the attenuation of the rightward shift of the curve and absence of lower inflection point (LIP) in rats exposed to high-stretch ventilation with 25 cmH_2O airway pressure when vagotomized (HV_{25}-vago, grey triangles) or pretreated with NK-1 receptor blockade (HV_{25}-SPB, grey circles) in comparison with non-pretreated rats (HV_{25}-sham, black circles). Rats with low-pressure ventilation are also represented for comparison (LV, white circles). *B* and *C*, comparison of lung weight indexed to body weight (*B*) and lung myeloperoxidase activity (MPO; *C*) in rats submitted to low-pressure ventilation (LV), high-stretch ventilation with 25 cmH_2O airway pressure without (HV_{25}-sham) or after vagotomy (HV_{25}-vago) or pretreatment with NK-1 receptor blockade (HV_{25}-SPB). For box plots, the top and the bottom of each box represent the 75th and 25th percentiles; median values are represented by thin horizontal bars.

mechanical ventilation often leads to excessive alveolar stretch in some lung regions.

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Author contributions

The experiments were carried out in the laboratory of respiratory pathophysiology (director Professor Jammes) UMR MD2 P2COE, in the Faculté de Médecine Secteur Nord, Marseille. All co-authors have contributed to the conception and design of experiments, or analysis and interpretation of data. They all helped to draft the article or revise it critically for important intellectual content and finally approved the version to be published.

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